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# RETARDATION OF CELL DIVISION BY VITAMIN C IN PHYSIOLOGICAL CONCENTRATIONS

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## 1. On the Mechanism of the Action of Ascorbic Acid in the Body

Although numerous studies have been carried out on the gross and microscopic changes occurring in tissues in vitamin C deficiency, the mechanisms by which the effects are produced are still largely unresolved.

A survey of the pathological changes leads to the conclusion that the primary effect of ascorbic acid deficiency is an abnormality in the intercellular material, or perhaps at the cell surface, leading to changes in the bones and small blood vessels. In cases of this vitamin deficiency, formation of cartilage and bone is stopped, and the junction of epiphysial cartilage and bone is weakened. The widespread occurrence of hemorrhages points to the failure of the cells in the vessel walls to adhere normally and retain their functions as closed tubes, impervious to erythrocytes. This is due to failure in the formation and maintenance of intercellular materials and consequent weakness of the tissue (Wolbach and Bessey, 1942). The phenomena are of considerable interest to the cellular physiologist for they point clearly to some relation between cell structure and pathology, arising from a biochemical deficiency. Some of these questions of cellular action have been covered in a review by Reid (1943).

It appeared to be of some value to investigate the influence of l-ascorbic acid on cell division, because of the likelihood of some action on the cell surface, which might alter conditions there so as to produce an effect on cell division which can be measured quantitatively by known methods (Shapiro, 1941). Results to date indicate that ascorbic acid does not play a major role in tissue respiration since vitamin C depleted tissues show little reduction of oxygen consumption, and but a small increase when the vitamin is added (Stotz and Harrer, 1937). Stark, Gordon and Christensen state (in Elvehjem and Wilson, 1939), "it must be recognized that there has been assigned to ascorbic acid no respiratory function in animal physiology." This does not exclude the possibility that vitamin C might still play a minor role in cell oxidations, at the same time that it determines the form of the histological picture.

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Wolbach and Howe (1926) confirmed the fact that in scorbutus vitamin C is the only factor necessary for producing normal intercellular material. The deposition of homogeneous bone matrix was observed to make its appearance within a day of administering vitamin C to scorbutic guinea pigs. An inadequate ascorbic acid level in the body affects the collagen of fibrous tissues, bone dentine and cartilage matrices, and all non-epithelial cement substances, including that of the vascular endothelium (Wolbach, 1937). Another line of evidence hinting at the importance of vitamin C in cell division is that adduced by Phillips *et al.* (1940 and 1941). Ascorbic acid is concerned with the production of virile sperm in the bull, and is involved in the early phases of reproductive processes in the cow. Vitamin C administered to impotent bulls led to an elevation of the vitamin C level in both semen and blood plasma, and to a corresponding improvement in the breeding potency of the bull.

### 2. Agents Affecting the Rate of Cell Division

The rate of cell division may be altered by a variety of operations, e.g., by influencing the metabolic activity of the cell, the viscous and other intracellular forces, the cell surface, or by operating on a combination of these factors. Agents like cyanide, which profoundly depress cell respiration, will completely inhibit the cell division of the sea urchin egg. Calcium, on the other hand, will accelerate or retard cell division depending upon its concentration, and appears to act primarily on the cell surface (Shapiro, 1941). An absence of calcium leads to a disintegration of the embryo, occasioned by a falling apart of the cells (Herbst, 1900), and a general appearance of softness. With adequate calcium, the rapidly changing form of the embryo passes through its normal configurations, whereas with excess calcium, the rate of cell division is retarded. In this manner, if an effect on cellular metabolism can be eliminated, the influence upon the cell surface may be studied by observing the effect on the rate of cell division. The mechanism may be either a physical or physico-chemical one; in the case of the sea urchin egg, it was suggested that the calcium may combine quantitatively with protein at the cell surface according to a mass-action relationship, and thus alter the spatial relationship between these molecules in the surface and so change its mechanical properties (*cf.* also Zweifach, 1940).

### 3. Vitamin C Content of Normal and Malignant Tissue

Since cancerous tissue represents an abnormal growth with numerous cell divisions in a localized area, it is relevant to examine the results of studies of the effect of ascorbic acid administration and determinations of ascorbic acid content in such tissues.

Yovarsky *et al.* (1934), investigating the vitamin C content of various human organs, arrived at a range of from about 0.55 mgm. per gram for adrenal tissue, to about 0.40 mgm. for heart tissue, with wide individual variations. Musulin and collaborators (1936) using a dye titration method, found 0.61 mgm. ascorbic acid per gram of Philadelphia No. 1 rat sarcoma, with very little necrosis; 0.04 mgm./gm. in a human large liver carcinoma with severe necrosis; and 0.18 mgm./gm. in a human benign uterine tumor with slight necrosis.

Arloing *et al.* (1935) injected intravenously various dyes combined with vitamin C and ferrous and ferric chlorides. Occasional regressions of rabbit testicular tumors were observed, and in some human cancers of the tongue, tonsil, stomach and uterus, a retardation of growth and improvement in general condition. Severe edema was often produced near the tumor.

Boyland (1936) found that the indophenol-reducing potency of the Dael and Biltris transplantable guinea pig sarcoma is much reduced when the animal is maintained on a scorbutic diet. In this respect it behaves like normal tissues. When injected into scorbutic guinea pigs, ascorbic acid is selectively absorbed by the tumor tissue as well as by those tissues normally containing ascorbic acid. Ascorbic acid was found by Andervont and Shimkin (1939) to prevent the appearance of hemhorrage and ensuing regression of transplanted tumors treated with bacterial filtrate. They postulated that the bacterial filtrate lowered the ascorbic acid content of the tumor, and weakened its capillaries, with resultant hemorrhagic extravasation. Minor and Ramirez (1942) working with hospital patients, reported that the daily utilization of vitamin C averaged 67 mgm. for noncancerous patients, 68 mgm. for a patient with localized cancer, and 125 mgm. for patients with metastatic cancer. They suggested an accelerated usage of vitamin C by carcinomatous tissue. A. F. Watson (1936) had earlier found that the vitamin C reserves in the tissues of guinea pigs on a scorbutic diet are exhausted more rapidly if the animals are supporting rapidly growing tumors. Carruthers and Suntzeff (1942) observed that the ascorbic acid content calculated on the basis of weight of original tissue is significantly less in carcinomas than in the epidermis of normal mice, benzene-treated controls, or methylcholanthrene-treated mice. Although they found further that the ratio of ascorbic acid to nucleoprotein phosphorus was nearly the same for all groups, it does not abolish the significance of the differential ascorbic acid values found on the basis of wet weight. Robertson (1943), however, determined the ascorbic acid content of 22 tumors (of rat and mouse) and of comparable normal tissues. The ascorbic acid content of the tumors ranged from 15 to 70 mgm. per 100 gm. of fresh tissue. The ascorbic acid content of the tumors was not related to that of the tissues of origin. Robertson also concluded that no correlation between ascorbic acid concentration and rate of growth was apparent. Vogelaar and Erlichman (1937), studying the *in vitro* growth of Crocker mouse sarcoma 180, decided that vitamin C stimulates both the emigration of cells and the frequency of cell division, but furnished no quantitative measurements in substantiation.

In assessing the above results it should be borne in mind that apart from ascorbic acid, 2:6 dichloroindophenol will be non-specifically reduced by substances like stannous and ferrous salts, sulfites, sulfhydryl compounds, sulfides, thiosulfates, reductinic acid and "reductones," the latter formed by fermentation, or by splitting of sugars by heat at a suitable pH, and especially in the presence of protein (Farmer, 1944). These substances may have a structure similar to ascorbic acid with an aldol type of condensation between carbohydrate and protein derivatives.

### 4. Experimental Procedure

The following technic was adopted in order to measure the time required by fifty per cent of a population of cells to undergo first division in embryological development. Crystalline ascorbic acid (Eastman Kodak Co., Rochester, N. Y.)

was used and the solutions made up shortly before use.[1] The eggs of the sea urchin, *Arabacia punctulata*, were handled as in previous studies, e.g. (Shapiro, 1941). All eggs used in any given experiment were obtained from a single urchin in order to obtain the most homogeneous cell population available. 3 ml. egg suspensions were pipetted into a pair of 300 ml. Erlenmeyer flasks, forming a shallow layer on the bottom of each flask, to facilitate gaseous interchange. The flasks were immersed in a constant temperature bath at 26.1° C. ± 0.002° C., controlled by a thermionic relay. Thermostatic control is necessary since the rate of cell division is known to be markedly affected by temperature (Loeb and Wasteneys, 1911; Hoadley and Brill, 1937). After temperature equilibration (about ten minutes), the eggs in both control and experimental flask were fertilized at the same time by the addition to each flask of 6 drops of a previously prepared suspension of sperm in sea water, and both flasks were shaken while

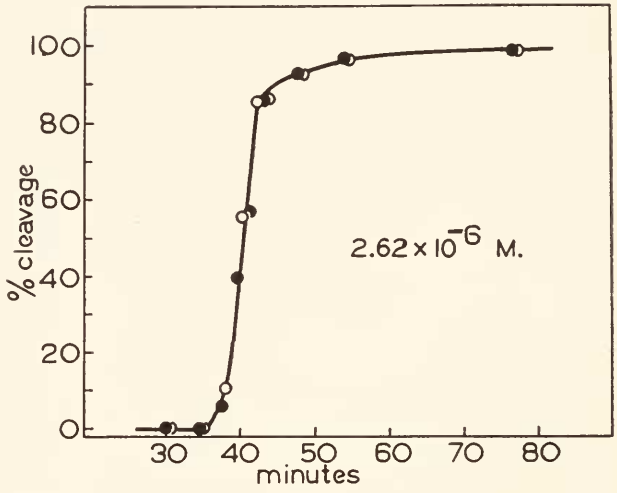


FIGURE 1. Cleavage curve showing the absence of effect at low concentrations of vitamin C, and the superposition of the two curves. Open circles, control eggs in sea water; closed circles, eggs in sea water containing $2.62 \times 10^{-6}$ molar vitamin C.

immersed, to distribute the sperm. After this the flasks were clamped in a stationary position. Oxygen was passed through sea water, and then over the shallow layer of eggs in each flask, to insure adequate oxygenation throughout the experiment. At regular intervals samples of the eggs were removed, and fixed for subsequent counts of per cent cleavage (cf. Fig. 1). At much later intervals, after the experiment, the eggs were examined to check for normal developments.

Where small effects on cleavage rate are encountered, it is essential to observe three precautions outlined above, in order to obtain reproducible results. Temperature must be controlled, adequate oxygenation insured, and the curve of cleavage

[1] Owing to the buffering capacity of sea water and the small amounts of vitamin C used, it is unlikely that the pH of the sea water was reduced from its normal value (approximately 8.1) to the region where pH is known to exert an influence on cell division (below pH 5.8, Smith and Clowes, 1924).

as a function of time determined by an adequate number of samples, and plotted. Simple observation of eggs in syracuse watch glasses at the side of the aquarium may be satisfactory where gross retardation of many minutes is observed, but for optimal quantitative results the experience of the writer has led to the adoption of the above technic. Since a population of cells is being observed, with a statistical distribution of the time at which the cell divides, sigmoid curves of the types shown in figures one and two are obtained. The slope at the midpoint is variable, and depends upon the condition of the eggs, as well as the time in the breeding season when they are used.

## 5. Results

The data (Fig. 2 A–D) demonstrate clearly that vitamin C added to a suspension of eggs will delay cell division at all concentrations above a minimal one. The slowing down of cleavage is more effective the higher the concentra-

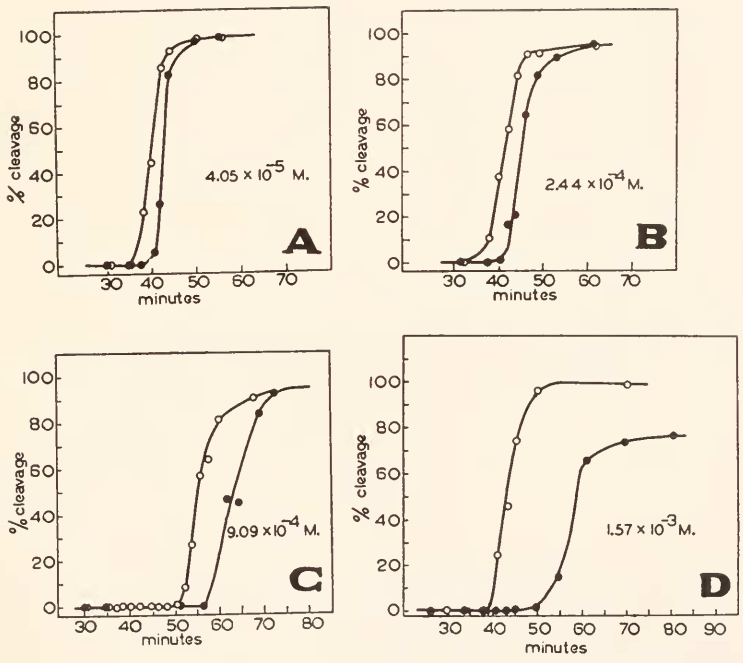


Figure 2. A, B, C and D show the increasing retardation of cell division with increasing concentration of vitamin C. The concentrations are given to the right of each pair of curves. Open circles, controls; closed circles, eggs in sea water with vitamin C.

tion, but as evidenced in Figure 2 A–C, the cells are not prevented ultimately from undergoing the same total per cent cleavage as the controls. At the concentration shown in Figure 2 D ($1.57 \times 10^{-3}$ molar), not only is there a considerable delay in cleavage, but it appears that in approximately 22 per cent of the cells, cleavage is entirely inhibited. At very high concentrations of vitamin C the agent

becames toxic, and cell division can be inhibited in 100 per cent of the cells; moreover, the reaction leading to elevation of the fertilization membrane is likewise prevented. No concentration was found at which cleavage was accelerated.

In Figure 3, the amount of delay is plotted as a function of vitamin concentration. In the range studied (up to 277 micrograms per ml.), the relationship is a linear one, except at the very low concentrations, in the region of 0 to 5 micrograms per ml.

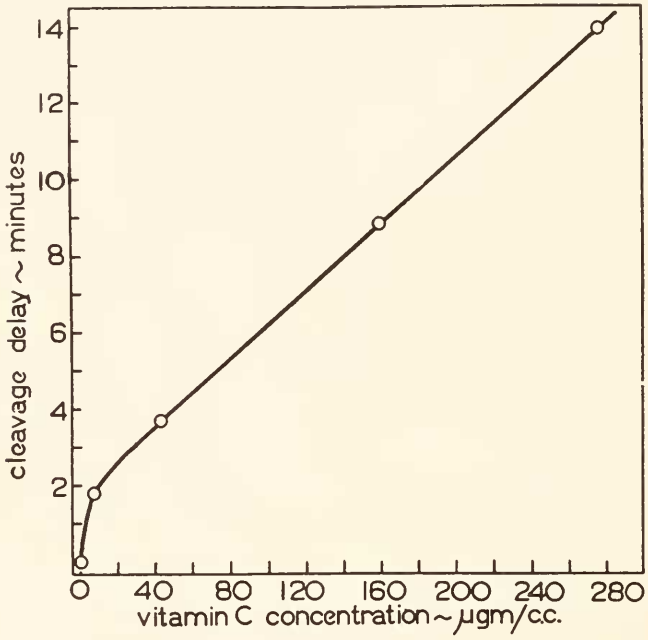


FIGURE 3. Concentration effect of vitamin C in retarding cell division.

## 6. SUMMARY

a. Eggs of the sea urchin, *Arbacia punctulata,* were exposed to concentrations of vitamin C in sea water, varying from $2.62 \times 10^{-6}$ molar to $1.57 \times 10^{-3}$ molar. The rate of cell division was determined by measuring the time required for 50 per cent of the cells to go through first cleavage.

b. Beginning with approximately $10^{-5}$ molar solutions, a definite retardation of cell division was observed. At high concentrations, complete inhibition of division occurred. At an intermediate concentration, the total percentage of cells dividing may be reduced from 100 per cent to some smaller figure.

c. Although cleavage was retarded in the presence of vitamin C, it appeared to be normal in other respects, with the exception of irregular cleavages at the high concentrations.

d. The slowing down of the rate of cell division appears to be roughly a linear function of the concentration, beyond a minimal concentration.

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